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METHODS FOR RAPID IDENTIFICATION OF VIRUSES

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16. ABSTRACT

The progress made in the last few years in methods for direct detection of viruses in clinical samples, without the need for in vitro culture, suggests that they will be applicable to at least some types of environmental samples. The methods for detecting viral antigen include: radioimmunoassay, enzyme immunoassay, agglutination tests, fluorescent antibody techniques and electron microscopic procedures. The use of probes for detecting viral nucleic acid has recently sparked considerable interest. The current status of these methods and potential means for improving them are presented in this report.

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I. Introduction

The need for rapid viral diagnosis in clinical situations is well-recognized, in that a diagnosis of viral infection will influence patient management. This includes both appropriate treatment and institution of infection control measures in hospital environments. Although the availability of antiviral agents is limited at present, specific diagnosis can eliminate unnecessary treatment. Rapid diagnosis in this situation usually means the ability to obtain results the same day a patient specimen is submitted, or the next day at the latest.

For environmental samples, there has long been a need for rapid methods to determine if viruses are present in samples such as food and drinking water. The recent findings that viruses, especially rotavirus, can be isolated from treated, municipal drinking water[1,2,3] makes it more important that a technology for rapid detection of viruses be developed. Rotavirus is the major cause of pediatric hospitalization for acute diarrheal disease, and in developing nations rotavirus infections are a major cause of infant mortality.

There are reports of waterborne outbreaks of gastroenteritis with suspected rotavirus etiology,[4,5,6] and the knowledge that rotaviruses survive water treatment[7] and have been isolated from drinking water supports the concept of waterborne rotavirus infections.

Monitoring viruses in other environmental samples, eg. sewage effluents, sludge, and source water is also becoming more necessary, but the need for a rapid result is usually less important. The major interest in these samples is virus survival, which can be measured by infectivity assays.

The major problems in developing rapid techniques for monitoring any environmental sample for viruses is the sensitivity required and the large number of viruses, in terms of varieties, that potentially require monitoring. The types include those in the enterovirus group, adenoviruses, hepatitis viruses, rotavirus, Norwalk virus, Norwalk-like viruses, and possibly astroviruses and caliciviruses.

Many of the viruses within the virus groups do not share common antigens and there is little or no cross-reactions among the groups. Thus, it would not be practical to monitor for all of these viruses by use of immunochemical methods. Rather, selection of an indicator virus would be necessary if a monitoring technique based on antigen detection by immunoassay was to be developed. The same would be true if other type-specific detection methods, such as nucleic acid hybridization, were used.

In addition to the technical difficulties above, techniques for virus detection which are not based on infectivity do not distinguish between infective and non-infective viruses. This is important for environmental samples which have undergone treatment that inactivates virus, but retain inactivated virus particles that will react in an immunochemical test. For this reason, for any types of monitoring test developed, infectivity data would need to be supplied.

Even with these limitations, the progress made in the last few years in methods for direct detection of viruses in clinical samples, without the need for culture _in vitro_, suggests that the methods will be applicable to at least some types of environmental samples. The current status of these methods and potential means for improving them will be the emphasis given here.

II. Viral Antigen Detection

A. Radioimmunoassay

Radioimmunoassay (RIA) techniques for detecting viral antigens, especially hepatitis B surface antigen, have been used for some time. Although RIA has been replaced, for most assays, by enzyme-immunoassay (EIA) for detection of viral antigens, not all RIA tests have been converted to EIA.

RIA tests can be performed in two general formats, 1) competitive binding in solution, and 2) solid phase RIA. The competitive assay was the one originally described by Halow and Berson,[8] and was used in a number of assays before Catt and Tregear[9] described solid-phase assays in 1967. The advantage of solid-phase assays is that it provides an easily controlled means for separation of antigen-antibody complexes.

B. Enzyme Immunoassay

Enzyme-immunoassay (EIA) evolved as a result of the findings by Nakane and Pierce[10] that antibodies could be labelled with enzymes for use in histochemical staining procedures, and by Catt and Tregear[9] who described solid-phase radioimmunoassays (RIA). The substitution of enzyme labels for radioactive ones in the solid-phase RIA resulted in solid-phase EIA tests for human chorionic gonadotropin by Van Weeman and Schuurs,[11] and for IgG detection by Engvall and Perlmann.[12] The latter authors coined the term "enzyme-linked immunosorbent assay (ELISA)" for solid-phase EIA tests.

Initial solid-phase EIA tests were not as sensitive as the corresponding RIA, but improvement in enzyme labelling techniques have made the two types of assays comparable for detecting a number of antigens and antibodies. In some systems where RIA and EIA have been directly compared for viral antigen

detections there is little difference in the sensitivity or specificity of the two assays.[13]

The advantages of enzyme labels over radioactive ones are mainly convenience in use, in that the labelled immunoreagents are stable for long periods, and the precautions and disposal procedures required for radioisotopes are unnecessary. In addition, the use of chromogenic substrates for the enzyme labels permits visual interpretation of test results in some cases. The only real disadvantages of EIA tests are the loss of antibody reactivity that may result from conjugation to enzymes, and the limits of substrate detection. For example, use of enzymes that have molecular weights higher than that of IgG molecules such as B-D-galactosidase (mw 540,000 daltons) can cause steric hindrance of antibody activity.[14] With regard to limits of substrate detection, improvement of enzyme detection by use of fluorogenic, luminescent, or radioactive substrates (reviewed by Yolken[15]) has been proposed.

The general principles of EIA tests and details of earlier studies have been reviewed a number of times[15-18], and will not be repeated in detail here. Rather, the major emphasis will be on current developments in EIA methodology and the application of EIA to detection of viral antigens in clinical and environmental samples.

C. Agglutination Tests

Because there is no need for a separation step, agglutination tests based on specific antibody immobilized on polystyrene (latex) particles offers an attractive alternative to EIA or RIA tests. In addition, antibody-coated latex particles are stable for long periods, and the tests require only 15-30 minutes for completion. The major problem to date in use of these tests for

direct detection of viruses is lack of sensitivity. A latex agglutination test for diagnosis of rotavirus infection is available, which is useful for rapid screening of fecal samples, but is less sensitive than EIA for detection of rotavirus in the same samples.[19] A test such as this may be useful for monitoring sewage and virus concentrated from water, if the test sensitivity could be improved. The judicious application of monoclonal antibodies and better techniques for immobilizing antibody on the particles may give latex agglutination techniques the sensitivity required for detecting a variety of virus types. At this time however, the techniques have not been sufficiently developed for general use in rapid viral diagnosis.

D. Fluorescent Antibody Techniques

For rapid diagnosis of some virus infections, direct detection of virus in clinical specimens by use of fluorescent antibodies (FA) remains a highly useful technique. The technique is particularly applicable to diagnosis of respiratory virus infections, and permits diagnosis within 2-3 hours after the specimen is received. In comparison to isolation in cell culture, the FA technique is approximately 96% sensitive and 98% specific for diagnosis of respiratory syneytial virus, influenza virus type A, and parainfluenza viruses types 1-3.[20] It is less sensitive for diagnosis of herpes simplex in skin lesions (88.7%), and specificity may also be a problem.[20] Use of monoclonal antibodies for herpes simplex diagnosis by FA does not increase the sensitivity.[21]

The limitations on the use of FA for viral diagnosis is that it requires cells containing viral antigen and is not useful for detection of virus in

samples such as serum or feces. For the same reason, the FA technique is not useful for direct detection of viruses in environmental samples.

In combination with cell culture, the FA technique can be used to shorten the time required for virus identification. This has been utilized for identification of poliovirus in experimentally contaminated water,[22] and in naturally occurring rotavirus in sewage.[23]

E. Electron Microscopy

Electron microscopy has been used for direct observation of virus particles in clinical samples for some time. The advantage of the technique is that all types of viruses can be recognized. Specific identification can usually be made by morphological characteristics alone, but immune electron microscopy can be used if necessary. The limitation of the technique is sensitivity, in that a minimum of 10^6 viral particles are required. The technique has been used to detect adenoviruses immersed in water,[24] but has not been used effectively for detecting naturally occurring viruses in water.

III. Detection of Viral Nucleic Acid

An alternative to antigen detection for rapid viral diagnosis is the detection of viral nucleic acid. The potential advantages of this technique are high specificity due to the requirement of complementarity for nucleic acid strand reassociation with the sample and DNA probe, and high sensitivity because of the high avidity of complementary strands for each other. The disadvantages of the current procedures for viral nucleic acid detection by hybridization are the use of radioactive probes, the time required to obtain results (18-24 hr or more) for many of the assays, and the complexity of the procedure relative to EIA or RIA tests.

The radioactive isotope most often used is ^{32}P, which in addition to hazards in handling, has a short half-life. To eliminate this problem, biotinylated probes have been developed, which, when combined with biotin-avidin-enzyme complexes, provide stable reagents and also reduces the assay time to approximately 5 hr. The general technique for preparation and use of biotinylated probes has been published,[25] and applied to probes for herpes simplex virus.[26] However, a comparison of ^{32}P-labeled probes and biotinylated probes for detection of viral nucleic acid in clinical specimens has not yet been reported.

Probes to detect RNA viruses have also been developed, including ones for poliovirus.[27] Whether the sensitivity will be sufficient for detecting virus in environmental samples remans to be demonstrated. The potential high specificity of nucleic acid probes would appear to limit their use as a broad spectrum method for monitoring virus contamination of water or other samples, as discussed above for antigen immunoassay techniques. However, as discussed more fully below, the probes described for poliovirus[27] appears to be broadly

reactive with other enterovirus types. This might make them more suitable for a general monitoring technique.

IV. Methodology

A. Enzyme Immunoassay

1. Design of Enzyme Immunoassays

a) Solid-phase Assays

The solid-phase or heterogeneous EIA requires immobilization of antigens or antibodies to a solid surface as a means of separating antigen-antibody complexes. Solid-phase surfaces used to immobilize antigens or antibodies have for most applications been polystyrene beads, tubes, and wells of microtiter plates, or wells of polyvinyl chloride microtiter plates. Coupling of proteins to these surfaces is usually done by passive adsorption. More recently, adsorption of antigens or antibodies to nitrocellulose membranes has been adapted to detection of viruses by EIA.[28] Covalent linkage of antibodies or antigens to a variety of surfaces, including porous glass[29], nylon[30], cellulose[31], agarose[32], and polyacrylamide[33], has been described.

The majority of solid-phase EIA tests that have been found to be useful for detecting viral antigens and antibodies utilize plastic plates or beads, with antigen or antibody passively adsorbed to the solid phase. In situations where a given antigen does not readily attach, antibody to the antigen is applied first. For some antigens, non-specific adsorbents such as poly-L-lysine have been used to enhance antigen adsorption, or the C1q component of complement to capture antigen-antibody complexes.[15] Most of the assays described are of the non-competitive type, although a number of competitive assays have been described. The disadvantage of many competitive assays for antigen detection is that labelled antigen is used which is usually more difficult to prepare than labelled antibody. With the advent of monoclonal antibodies, competitive assays for specific antibodies are becoming more common.

The lower limit of sensitivity for detecting antigen by EIA in most studies is approximately 100 pg to 1 ng, although lower limits have been described. This level of sensitivity is sufficient to detect virtually all culture-propagated viruses, but is not always sensitive enough to detect antigen directly in clinical specimens.

The types of formats most often used are shown in Table 1.

b) Homogeneous Assays

To avoid the need for separation of antigen-antibody complexes, homogeneous EIA tests were developed.[34] Homogeneous assays are based on reaction of antigen with an antibody-enzyme complex. This results in steric hindrance of the enzyme, which cases a decrease in product after reaction with enzyme substrate. The major advantage of homogeneous assays is that they do not require the separation and washing steps required in heterogeneous assays. The major disadvantages of this type of EIA is that it has been difficult to apply it to detection of high molecular weight antigens with the degree of sensitivity required. Thus, the homogeneous assay has been used mainly for detection of hormones, drugs, and other low-molecular weight substances. Means to improve the sensitivity of homogeneous assays have been devised[15] but have not been widely used. Thus, the applications discussed here will be limited to EIA tests of the solid phase type.

2. Enzymes and Substrates

Most EIA formats require covalent coupling of enzymes to antibody or antigen. A number of enzymes and coupling techniques have been tried.[15] The most consistent reuslts have been obtained with horseradish peroxidase coupled by use of periodate,[35] and alkaline phosphatase coupled by use of

glutaraldehyde.[36] Most of the assays found to be clinically useful in diagnosing infectious diseases use chromogenic substrates, although fluorogenic and radioactive substrates have been described,[15,37] as have luminescent ones (review by Seitz[38]). Other enzymes, such as β-D-galactosidose and glucose oxidase have also been used. However, the high carbohydrate content of peroxidase permits use of the periodate method for coupling, which is more efficient than methods using bifunctional reagents. Several chromogenic substances have been commonly used in combination with the substrate for peroxidase, hydrogen peroxide. These include a O-phenylenediamine (OPD), 5-aminosalicylic acid, and 2,2'-azinodi (3-ethylbenzothiazoline-6-sulphonate (ABTS). All give comparable sensitivity in detecting peroxidase. Several other enzymes have been used less frequently for heterogeneous EIA, including acetylcholinesterase, adenosine deaminase, catalase, and urease. Catalase, which has a specific activity of 40,000 units/mg would appear to be preferrable to peroxidase, which has a maximum specific activity of 4,500 units/mg. However, its product absorbs in the ultraviolet range, which is not always satisfactory for biological materials. Further details on the use of various enzyme and substrate systems for EIA are available in a recent review.[39]

A more recent development in EIA which has found application in diagnostic microbiology is the use of avidin and biotin.[40] The tests is based on the high affinity complex formed between biotin and avidin. The most common method is to use specific antibody labelled with biotin; the indicator system is enzyme-labelled avidin. A recent adaptation of this method using biotinillated beta-lactamase in combination with avidin was effective for detecting rotavirus antigen.[41]

3. Solid Phase Supports

In addition to antibody affinity and the sensitivity of the indicator system used, the sensitivity of many antigen detection systems depends on the amount of antibody that can be effectively immobilized on a solid phase. Methods for immobilization of antibody on plastic surfaces are usually based on simple adsorption, although convalent-linking methods have been utilized. The amount of immunoglobulin that can be immobilized on various plastics varies and has been reported to be from 0.9 to 5.7 ng/mm^2 by use of passive adsorption.[42] If covalent coupling is used, the quantity of immunoglobulin immobilized may be increased from 100 to 590 ng/mm^2. Increasing the amount of antibody immobilized by use of covalent techniques may increase the EIA sensitivity to some degree. However, it has been noted that increasing the concentration of antibody for coating surfaces by passive adsorption beyond 10 ug/ml does not give an increase in immunoassay sensitivity. This is apparently due to desorption of antibody from the plastic surface, and steric hindrance of antibody that is adsorbed.

Solid-phase surfaces other than plastic tubes, beads, or particles, such as porous glass[29] have been used to immobilize antibody. However, the majority of EIA tests for microbial infections use either plastic plates or beads. Another development mentioned above that may be applicable to a variety of viral antigens and antibodies is the use of nitrocellulose membrane discs as a solid phase. This was successfully developed as a visual-readout method for detecting adenovirus antigens and antibodies.[28]

4. Immunoreagents

The use of antibodies that are highly specific and have high affinity is the most critical aspect of most EIA techniques. An example of how the reagents used determines EIA effectiveness was shown for detection of Clostridium deficile toxin, where the sensitivity was increased from 58.6% to 95% by changing the immunoreagents used.[43] The diluents used for antigen preparation can also alter the sensitivity. Disrupting viruses with detergents or other chemicals may increase the sensitivity of some assays but decrease others.[15] A number of diluents not usually used in EIA tests was tested by Conroy and Esen[44] for adsorbing a plant protein to polystyrene. These included detergents, acids, alcohols and urea. Use of alcohols or urea increased the EIA substantially. Whether this would be applicable to microbial antigens remains to be tested.

The high number of monoclonal antibodies being developed to viral antigens may also improve immunoassay reliability. The use of monoclonal antibodies in EIA tests offers two potential advantages: 1) improved specificity due to the nature of monoclonal antibodies and, 2) improved sensitivity by allowing for clearer EIA cut-off values. Sensitivity could also be increased by increasing the amount of detector antibody used in an EIA. However, because monoclonal antibodies react with only one epitope of a given antigen, more than one monoclonal antibody may be needed to achieve the desired sensitivity. In practice, monoclonal antibodies have been used successfully in latex agglutination tests and also in immunofluorescence techniques.[45] Their use in EIA has been limited, but the number of ones described for microbial antigens to date suggest that applications in clinical diagnosis will be increasing, and the EIA tests that have been developed look promising. For

EIA detection of adenovirus group antigens in stools, monoclonal antibodies were as sensitive as polyclonal ones, and were more sensitive for detecting non-cultivatable adenoviruses (presumably enteric serotypes). All 12 stool samples positive by EM were monoclonal EIA positive.[46] For diagnosis of rotavirus infection, we have found that a monoclonal EIA was 100% sensitive and specific for samples from adults and neonates as well as young children (Herrmann, et al. 1985, submitted for publication). This was possible due to the high affinity and broad group specificity of the monoclonal antibody used.[47]

Monoclonal antibodies to hepatitis B surface antigen have been described, and were found more sensitive than polyclonal antibodies for detection of viral antigen in serum by RIA.[48]

B. Nucleic Acid Hybridization

The principle of nucleic acid hybridization is that a single-stranded DNA probe specific for a given viral DNA (or RNA) will recognize and bind in a complimentary manner to denatured (single-stranded) DNA or RNA, from the target virus. In practice, this reaction is usually demonstrated by use of radiolabeled probes and nitrocellulose filters as a solid phase for the hybridization. The procedures, though tedious and time-consuming compared with EIA, have been sufficiently developed such that they can be routinely done in many laboratories.

The general procedures, condensed from several methods reported[26,49-52], are shown in Table 2. Note that the "sandwich" method requires that two separate probes (which need to be derived from two nonoverlapping regions of the genome) be used.

For single-stranded RNA viruses, molecular probes from DNA clones of the viral RNA genome are used. One problem that occurs with single-stranded RNA is that it is inactivated by some of the procedures used to extract DNA, and many clinical materials contain high levels of ribonuclease activity. To circumvent the latter problem, ribonuclease inhibitors need to be added.[26]

The major problem at the moment for application of nucleic acid hybridization to routine virus detection is the relative complexity of the procedure. Complete details can be found elsewhere[26,49-52] and will not be repeated here for the various viruses. One example, though, that for detection of cytomegalovirus, provides an example of what is involved. To detect cytomegalovirus in urine specimens, the procedure requires centrifugation of the sample for 1 hr at 20,800 rpm, treatment of the pellet with detergent and proteinase, extraction of the mixture once with phenol-chloroform-isoamyl alcohol and twice with ethylether, followed by precipitation with ethanol.[52] This is for extraction of the DNA only, prior to hybridization procedures. This is not a problem for research laboratories, but is not applicable to clinical laboratories for routine screening.

V. Applications in Rapid Viral Diagnosis

A. Virus Detection in Clinical Samples

1. Enzyme Immunoassay

The interest in EIA methods for rapid diagnosis of viral infections has been high, because of the time and expense required for isolation of the agents in cell culture. Further, some viruses cannot be cultivated or are difficult to cultivate. The latter includes Hepatitis A and B viruses, and rotavirus. Tests for Hepatitis B surface antigen and e antigen have been commercially available for some time, have been extensively evaluated, and need not be elaborated on here. Detection of Hepatitis A antigen by EIA has also been reported,[53,54] but is not at this time commercially available. The sensitivity of the EIA developed by Mathiesen, et al.[53] was 77% (10/13) compared with immune electron microscopy (IEM). Locarini, et al.[54] were able to detect Hepatitis A in 85% (17/20) of samples positive by IEM, and found no false positive EIA reactions in fecal samples from patients with Hepatitis B or Non A-Non-B hepatitis virus infections.

In addition to detection by EIA of Hepatitis viruses, the EIA tests most frequently developed have been for respiratory viruses, herpes viruses, and gastroenteritis viruses. These and other viruses that have been detected in clinical samples by EIA are shown in Table 3.

a. Respiratory Viruses

Because antiviral agents are becoming available for some of the respiratory virus infections, rapid methods of diagnosis are essential for prompt treatment. Rapid diagnosis by EIA has been proposed for a number of respiratory viruses. Several have been described for diagnosis of respiratory syncytial virus. Specificity does not appear to be a problem with any EIA

reported, but the sensitivity is less than that found by culture. Compared with culture, sensitivities have been found to be 79.3% (23/29),[55] 60.9% (25/41),[56] 78.7% (37/47),[57] and 82.8% (77/93)[58].

Diagnosis of viral influenza by EIA has also been reported, with variable results. Compared with culture, Harmon and Pawlik[59] reported a sensitivity of 53% (21/40). A later report by Harmon, et al.[60] on an EIA using fluorogenic substrates gave a sensitivity of 87% (27/31). By use of a radioactive substrate, Coonrod, et al.[61] were able to detect influenza virus in nasal washes, but the maximum sensitivity at any given day of infection was 48% (12/25). A similar assay tested on samples from 12 volunteers gave sensitivities of 78 to 100%, depending on the day tested.[62] Tests for adenovirus in respiratory secretions have also been developed. Harmon and Pawlik[59] compared an EIA with tissue culture isolation and were able to detect by EIA 62% (13/21) of adenovirus-positive specimens.

b. Herpesviruses

The interest in sexually-transmitted herpes virus and the availability of treatment have led to the development of a number of EIA tests for rapid diagnosis of herpes infection. Most lack sufficient sensitivity to be used as a substitute for culture. Two evaluations of a commercial EIA for herpes genital infection (Ortho Diagnostic Systems, Inc.) have been reported. Morgan and Smith[63] found the test to be 71.9% (105/146) sensitive and 100% specific in 366 control specimens. Warford et al.[64] however, found the test to be only 52.5% (155/295) sensitive and 96.9% (834/860) specific. Some of the other EIA tests developed have given similar results. Lawrence, et al.,[65] developed an EIA which was 50.5% (94/186) sensitive and 99.1% (423/427) specific. An EIA reported by Grillner and Landqvist[66] was 75.9% (44/58) sensitive and 100%

specific. Two assays reported appear to have higher efficiencies. Nerurkar et al.[67], using a biotin-avidin EIA obtained a 95.6% sensitivity and a 91.4% specificity; Land, et al.[68], using a detergent-treated specimen obtained a sensitivity and specificity of 94%.

Development of EIA tests for other viruses in the herpesvirus group have also been reported. Ziegler[69] developed an EIA for varicella-zoster viral antigens which detected 8/8 culture positive specimens, and Pronovost, et al.[70] developed a chemiluminescent EIA for cytomegalovirus antigen which detected 9/11 culture-positive specimens.

c. Gastroenteritis and Enteric Viruses

The two most important gastroenteritis viral agents for which EIA tests have been developed are rotavirus and enteric adenoviruses. An EIA test for rotavirus was first developed by Yolken, et al.[71], and commercial assays are now available. A recent evaluation of two commercial products (Rotazyme, Abbott Laboratories, and Enzygnost, Behring) showed the sensitivity of Rotazyme to be 88% and Enzygnost, 98%.[19] The standard for comparison was electron microscopy (EM). Both EIA methods appear suitable for use if EM is not available, although Rotazyme is known to cause false-positive results in samples from neonates[72,73] and is insensitive in samples from adults (Herrmann, et al., 1985, submitted for publication).

Enteric adenoviruses (types 40 and 41) are difficult to isolate, therefore EIA methods would be preferable if the sensitivity was satisfactory. Preliminary results from Johansson, et al.[74] suggest that development of an EIA specific for enteric adenoviruses is possible.

Because of the large number of types within the enterovirus group, EIA tests have not been widely applied for these viruses. Type-specific tests

have been used to identify coxsackievirus A and B in stool specimens.[75,76] The test had an overall sensitivity of 70% (14/20) compared with tissue culture isolation. The detection limit was approximately 10^3 infectious units.

Another virus in the enteric group which may be important in environmental transmission is Norwalk virus. We have recently developed an EIA for this virus which is equally as sensitive as RIA for detecting Norwalk virus in stools of volunteers (Herrmann, et al., in preparation).

B. Virus Detection in Environmental Samples

Direct detection of virus in environmental samples by any culture-independent method has been very limited. Immunochemical methods for detecting viruses in water and sewage have been described for hepatitis B antigen,[77] but application of immunochemical methods to field samples have been limited to rotavirus detection in sewage. Steiman[78] tested a commercial rotavirus EIA (Behringwerke) on domestic sewage concentrated by adsorption to filters followed by ultracentrifugation, or by chemical precipitation. In samples concentrated by the first method, six samples positive by electron microscopy were also positive by the EIA. The chemical precipitation method was ineffective. In another study on rotavirus in sewage, the Rotazyme assay (Abbott Laboratories) was compared with a cell culture-immunofluorescence technique.[79] The EIA technique was not effective, giving a 23% false-positivity rate.

2. Nucleic Acid Hybridization

The goal of nucleic acid hybridization studies as applied to rapid viral diagnosis is to provide a more reliable means than immunoassays for detecting

viruses in clinical specimens. This goal has not yet been achieved, but a number of attempts have been made. The applications in diagnostic virology are summarized in Table 4.

The results of the assays, in general, have approximately the same sensitivity as EIA or RIA for the same virus. The assay for adenovirus in nasopharyngeal swabs gave the same specificity as the RIA, and detected 88% (22/25) of the RIA positive samples.[50] Culture was not done on all samples, so the true sensitivity of the method is unknown.

In one study on detection of cytomegalovirus (CMV) in urine and buffy coat specimens, the DNA-probe assay was 92% (22/24) as sensitive as culture and 88% (23/26) specific for urine specimens. In buffy-coat specimens, the assay was 93% (13/14) sensitive and 60% (32/53) specific.[52] In another study on CMV detection in urine specimens, DNA hybridization was 100% sensitive and specific on all specimens from 9 patients positive and 21 patients negative for CMV.[80]

An interesting finding was reported by Rotbart, et al.[27] for detection of enteroviruses in cerebrospinal fluid. The probe used was cloned cDNA to poliovirus type 1, which reacted with coxsackieviruses type A9 and B1, and echovirus 11 as well as poliovirus. The assay could detect 10^3 infectious units of poliovirus and 10^4 infectious units of the other enteroviruses. Virus could be detected in experimentally inoculated cerebrospinal fluid, but was not applied to clinical specimens. The broad specificity of the probe, however, suggests it might be useful for generic identification of enteroviruses.

Another RNA virus for which probes have been developed is influenza virus.[26] Specimens from naturally-occurring human infections have not yet

been tested, but virus could be detected nasal washings from infected monkeys.

Probes for a double stranded RNA virus, rotavirus, have also been described.[81] The sensitivity of the assay was 100% on 91 samples positive by electron microscopy, and correlated well with EIA on a number of other samples. By serial dilution of positive stool samples, the probe technique was found to be 10-100 times as sensitive as the EIA method used.

Use of probes for diagnosis of hepatitis B infection[82,83] has the advantage of quantitating virus in serum of carriers by direct detection of viral DNA, but does not offer any advantages over currently available immunological tests for hepatitis B antigens as a purely diagnostic test. The use of DNA probes for detection of viral DNA integration in cellular DNA of liver specimens, however, cannot be done by the immunological tests.[83]

Diagnosis of herpes simplex virus (HSV) infection is also an area where improved tests are needed, in that current EIA tests have not yet proven clinically useful due to a lack of sensitivity. Reports on use of DNA probes, which can detect about 1 pg viral DNA or 10^4 infectious units, indicate that the sensitivity and specificity is approximately that for EIA tests. In a comparison with culture isolation for detection of HSV infections, DNA detection was 80% sensitive for HSV type 2, and 90% for HSV type 1. The specificity was 100%.[84]

VI. Summary and Future Prospects

Although many of the current EIA tests for viral antigens have not realized their potential, there are reasons to believe this situation will improve. For detection of antigen, which offers a rapid and direct means of diagnosing microbial infections, the major problem has been lack of sensitivity. Increasing the sensitivity of polyclonal EIA tests by using more concentrated immunoreagents or more sensitive enzyme substrates has often resulted in a loss of specificity. From the reports available to date, it appears that use of the appropriate monoclonal antibodies may solve the problem of sensitivity for detecting many infectious agents in clinical samples. With the increasing number of monoclonal antibodies available for various viral antigens, we can expect that more of them will be utilized for EIA detection systems. If the affinities of monoclonal antibodies can be increased, the EIA tests may be sufficiently sensitive.

A similar situation exists with nucleic acid hybridization techniques in regard to sensitivity. Current tests require 10^3-10^5 infectious units of virus for detection, which is similar to that required for EIA.

For direct detection of virus in environmental samples, concentration of the samples would be needed with the present level of sensitivity of the culture-independent methods. Even with concentration, the ability to detect virus would depend on the sample tested. For example, the number of infectious viruses present in sewage may be in the range of 100 to 1000 or more per liter, whereas virus in river water might only contain 10 or less infectious units of virus.[85] For identification of specific virus types, such as rotavirus, a monoclonal antibody-based EIA test might be suitable for monitoring sewage and waste water for this virus. For a more broad-spectrum assay, use

of probes which react with more than one enterovirus type, such as the one for poliovirus[27] described above, may also be applicable to the same kind of samples. Further development and simplification of the nucleic acid detection methods would be required, however, to be practical for routine use.

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Table 1

Enzyme Immunoassays for Viral Antigen Detection

EIA type/procedure	
Non-competitive EIA	
Direct EIA	Indirect EIA
1. Antigen-specific antibody is attached to solid phase	1. Same as direct EIA
2. Test specimen containing antigen is added	2. Same as direct EIA
3. Enzyme-labelled specific antibody is added	3. Specific antibody (prepared in species different from that used in step 1) is added
4. Enzyme substrate is added	4. Enzyme-labelled antiglobulin specific for antibody used in step 3 is added
5. Substrate hydrolysis product is proportional to the amount of antigen present	5,6 Same as 4,5 in direct EIA
Competitive EIA	
Enzyme-Labelled Antigen Method	Enzyme-Labelled Antibody Method
1. Antigen-specific antibody is attached to solid phase	1. Antigen is attached to solid phase (may require use of specific antibody)

Table 1 (Continued)

2. Test specimen containing antigen is added	2. Test specimen containing antigen plus enzyme-labelled specific antibody is added
3. Enzyme-labelled antigen is added	3,4 Same as 4,5 in enzyme-labelled antigen method
4. Enzyme substrate is added	
5. Substrate hydrolysis product is inversley proportional to the amount of antigen present	

Table 2

Nucleic Acid Hybridization Assays

for Viral DNA

Assay type/procedure	
Single Probe Filter Hybridization	Sandwich Hybridization
1. Treat sample to release viral DNA (heat, organic solvent, or protease-salt)	1. Immobilize virus-specific probe No. 1 on nitrocellulose filter
2. Dry sample on nitrocellulose filter	2. Treat sample to release DNA; denature DNA by boiling
3. Heat for 2 hr. at 80°C to denature DNA on filter	3. Mix with labeled probe No. 2, react on filter containing probe No. 1 for 18 hr.
4. Hybridize with viral DNA-specific radiolabeled probe, 4-24 hr.	4. Detect by isotope counting or autoradiography
5. Detect by isotope counting or autoradiography	

Table 3

Enzyme Immunoassays for the Detection of

Viral Antigens in Clinical Specimens

Virus	Specimen	References
Adenovirus	Respiratory secretions Stools	13,59,86 74
Coronavirus	Respiratory secretions	87
Coxsackievirus	Stools	75,76
Cytomegalovirus	Urine	70
Hepatitis A virus	Stools	53,54
Hepatitis B virus	Serum	88
Herpes Simplex virus	Dermal lesions	63-68, 89
Influenza A virus	Respiratory secretions	59-62, 90
Parainfluenza virus	Respiratory secretions	13,91
Respiratory Syncytial virus	Respiratory secretions	13, 55-58, 92
Rotavirus	Stools	71,86
Varicella-Zoster virus	Dermal lesions	69

Table 4

Diagnosis of Viral Infections by

Nucleic Acid Hybridization

Virus	Specimen	References
Adenovirus	Respiratory Secretions	50
Cytomegalovirus	Urine	52,80
	buffy-coat cells	52
Enteroviruses[a]	Cerebrospinal fluid	27
Hepatitis B virus	Serum	51,82,83
	Liver	83
Herpes simplex virus	Dermal lesions	8
Influenza virus	Respiratory Secretions	26
Rotavirus	Stools	81

[a]Experimentally inoculated samples